User Manual

SMARTer™ Ultra Low RNA Kit for Illumina Sequencing User Manual

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Table of Contents

I. List of Components 3

II. Additional Materials Required 4

III. Introduction 5

IV. SMARTer cDNA Synthesis 7
- A. Requirements for Preventing Contamination 7
- B. General Requirements 7
- C. Sample Recommendations 8
- D. Sample Requirements 8
- E. PROTOCOL: First-Strand cDNA Synthesis 8
- F. PROTOCOL: Purification of First-Strand cDNA using SPRI Ampure Beads 10
- G. PROTOCOL: ds cDNA Amplification by LD PCR 10

V. Amplified cDNA Purification & Validation 12
- A. PROTOCOL: Purification of ds cDNA using SPRI Ampure Beads 12
- B. Validation Using the Agilent 2100 BioAnalyzer 12

VI. Covaris Shearing 15
- A. PROTOCOL: Covaris Shearing of Full-length cDNA 15

VII. Appendix A: PCR Clean Work Station Guidelines 16
- A. Equipment Needed in the PCR Clean Work Station 16
- B. PCR Clean Work Station Operation Instructions 16

VIII. Appendix B: Working with Cells 18
- A. PROTOCOL: Screen for Cell Culture Media 18
- B. Cell Preparation for First-Strand cDNA Synthesis 19

IX. Appendix C: PCR Optimization 20

X. Appendix D: Troubleshooting Guide 21

XI. References 22

List of Figures

Figure 1. Protocol Overview. 5
Figure 2. Flowchart of SMARTer cDNA Synthesis. 6
Figure 3. Electropherogram example results from Agilent 2100 Bioanalyzer 13

List of Tables

Table I: Sample Preparation Guidelines 9
Table II: Cycling Guidelines Based on Amount of Starting Material 10
Table III. Process Configuration Panel Set Up 15
Table IV: Media Screen Preparation Guidelines 18
Table V: Validated Media 18
Table VI: Troubleshooting Guide for SMARTer cDNA Synthesis & Amplification 21

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I. List of Components

SMARTer Ultra Low RNA Kit for Illumina Sequencing

The following components have been specifically designed to work together and are optimized for this particular protocol. Please do not make any substitutions. The substitution of reagents in the kit and/or a modification of the protocol may lead to unexpected results.

Box 1:

- 20 µl **SMARTer II A Oligonucleotide** (12 µM)
  5'–AAGCAGTGGTATCAACGCAGAGTACXXXXX–3'
  (X = undisclosed base in the proprietary SMARTer oligo sequence)
- 5 µl **Control Total RNA** (1 µg/µl)

Box 2:

- 20 µl **3′ SMART CDS Primer II A** (12 µM)
  5′–AAGCAGTGGTATCAACGCAGAGTACT$_{(30)}$N$_{-1}$N–3′
  (N = A, C, G, or T; N$_{-1}$ = A, G, or C)
- 20 µl **IS PCR Primer** (12 µM)*
  5′– AAGCAGTGGTATCAACGCAGAGT –3′
  *Please do not substitute. The oligo has been modified.
- 40 µl **5X First-Strand Buffer (RNase-Free)**
  250 mM Tris-HCl (pH 8.3)
  375 mM KCl
  30 mM $MgCl_2$
- 40 µl **dNTP Mix** (dATP, dCTP, dGTP, and dTTP, each at 10 mM)
- 10 µl **Dithiothreitol** (DTT; 100 mM)
- 20 µl **SMARTScribe™ Reverse Transcriptase** (100 U/µl)
- 1 ml **Nuclease-Free Water**
- 55 µl **RNase Inhibitor** (40 U/µl)
- 1 ml **Dilution Buffer**
- 1 ml **Purification Buffer (**10 mM Tris-Cl, pH 8.5)

Storage Conditions

- Store Control Total RNA and SMARTer IIA Oligonucleotide at –70°C.
- Store Dilution Buffer at 4°C.
- Store all other reagents at –20°C.

II. Additional Materials Required

The following reagents are required but not supplied. These materials have been validated to work with this protocol. Please do not make any substitutions because you may not obtain the expected results:

- **Single channel pipette:** 10 µl, 20 µl and 200 µl, one each
- **Eight channel pipette:** 20 µl and 200 µl, one each
- **Filter pipette tips:** 10 µl, 20 µl and 200 µl, one box each
- **One QuickSpin minicentrifuge for 1.5-ml tubes**
- **One QuickSpin minicentrifuge for 0.2-ml tubes**
- **One marker pen**
- **Small 1.5-ml tube rack**
- **One bag of 8-strip nuclease-free 0.2-ml thin wall PCR tubes with caps**
- **DNA-OFF Solution** (Takara Cat. No. 9036)

For PCR Amplification & Validation:

- **One dedicated PCR thermal cycler used only for first-strand synthesis**
- **Advantage® 2 PCR Kit** (Cat. Nos. 639206 & 639207)
  This kit has been specially formulated to provide automatic hot-start PCR (Kellogg *et al.*, 1994)—and can efficiently amplify full-length cDNAs with a significantly lower error rate than that of conventional PCR (Barnes, 1994).
- **High Sensitivity DNA Kit** (Agilent Cat No. 5067-4626)
- **IsoFreeze Flipper Rack** (MIDSCI Cat. No. TFL-20)
- **IsoFreeze PCR Rack** (MIDSCI Cat. No. 5640-T4)
- **Nuclease-free thin-wall PCR tubes** (0.2 ml; USA Scientific Cat. No.1402-4700)
- **Nuclease-free nonsticky 1.5-ml tubes** (USA Scientific Cat. No. 1415-2600)

For SPRI Bead Purification:

- **Agencourt AMPure PCR Purification Kit** (5 mL Beckman Coulter Part No. A63880; 60 mL Beckman Coulter Part No. A63881)

  Use this kit for the SPRI Purifications (Sections IV.F & V.A).
- **MagnaBot II Magnetic Separation Device** (Promega Part No. V8351)
  Use this stand for the first purification (Section IV.F).
- **Magnetic Stand-96** (Ambion Part No. AM10027)
  Use this stand for the second purification (Section V.A).
- **96-well V-bottom Plate (500 µL) (**VWR Cat. No. 47743-996)
- **MicroAmp Clean Adhesive Seal** (AB Part No. 4306311)
- **80% Ethanol**

For Sequencing Library Generation:

- **Illumina Paired-End DNA Sample Prep Kit** (Illumina Cat. Nos. PE-102-1001 & PE-102-1002)
- **Covaris Instrument and Related Materials for DNA Shearing**

III. Introduction

SMARTer cDNA Synthesis for the Illumina Sequencing Platform

The SMARTer Ultra Low RNA Kit allows high-quality cDNA synthesis starting from as little as 100 pg of total RNA or cells. The kit has been designed and validated to prepare cDNA samples for sequencing and quantitation with the Illumina HiSeq and Genome Analyzer sequencing instruments. The entire library construction protocol can be completed within two days (see Figure 1 for an overview of the protocol). SMART technology offers unparalleled sensitivity and unbiased amplification of cDNA transcripts, enabling a direct start from your sample. Most importantly, SMART technology enriches for full-length transcripts and maintains the true representation of the original mRNA transcripts; these factors are critical for transcriptome sequencing and gene expression analysis.

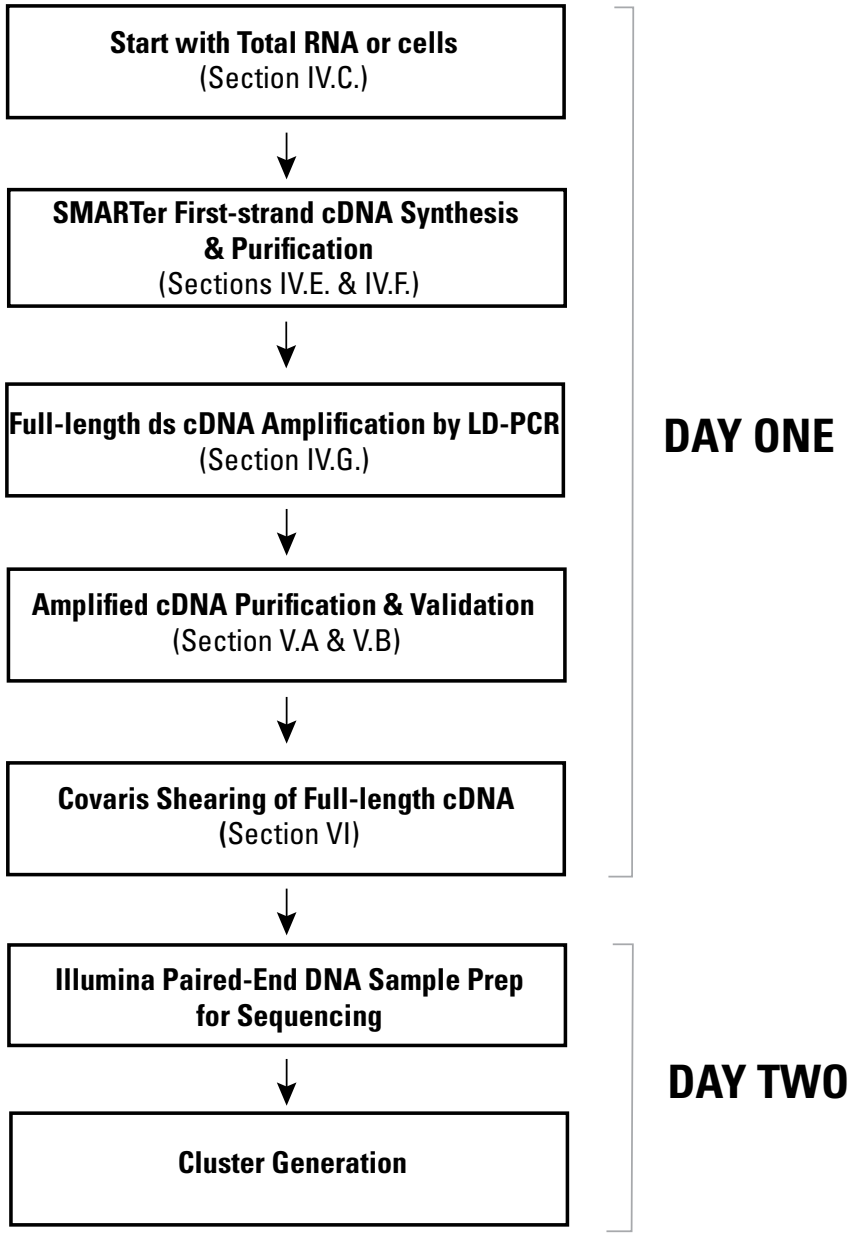


Figure 1. Protocol Overview.

III. Introduction, *continued*

SMARTer cDNA synthesis starts with picogram amounts of total RNA. A modified oligo(dT) primer (the SMART CDS Primer) primes the first-strand synthesis reaction (Figure 2). When SMARTScribe Reverse Transcriptase reaches the 5′ end of the mRNA, the enzyme's terminal transferase activity adds a few additional nucleotides to the 3′ end of the cDNA. The carefully-designed SMARTer Oligonucleotide base-pairs with the non-template nucleotide stretch, creating an extended template to enable SMARTScribe RT continue replicating to the end of the oligonucleotide (Chenchik *et al.*, 1998). The resulting full-length, single-stranded (ss) cDNA contains the complete 5′ end of the mRNA, as well as sequences that are complementary to the SMARTer Oligonucleotide. In cases where the RT pauses before the end of the template, the addition of non-template nucleotides is much less efficient than with full-length cDNA/RNA hybrids, thus the overhang needed for base-pairing with the SMARTer Oligonucleotide is absent. The SMARTer anchor sequence and the poly A sequence serve as universal priming sites for end-to-end cDNA amplification. In contrast, cDNA without these sequences, such as prematurely terminated cDNAs, contaminating genomic DNA, or cDNA transcribed from poly A^- RNA, will not be exponentially amplified. However, truncated RNAs with poly A tails that are present in poor quality RNA starting material will be amplified yielding shorter cDNA fragments.

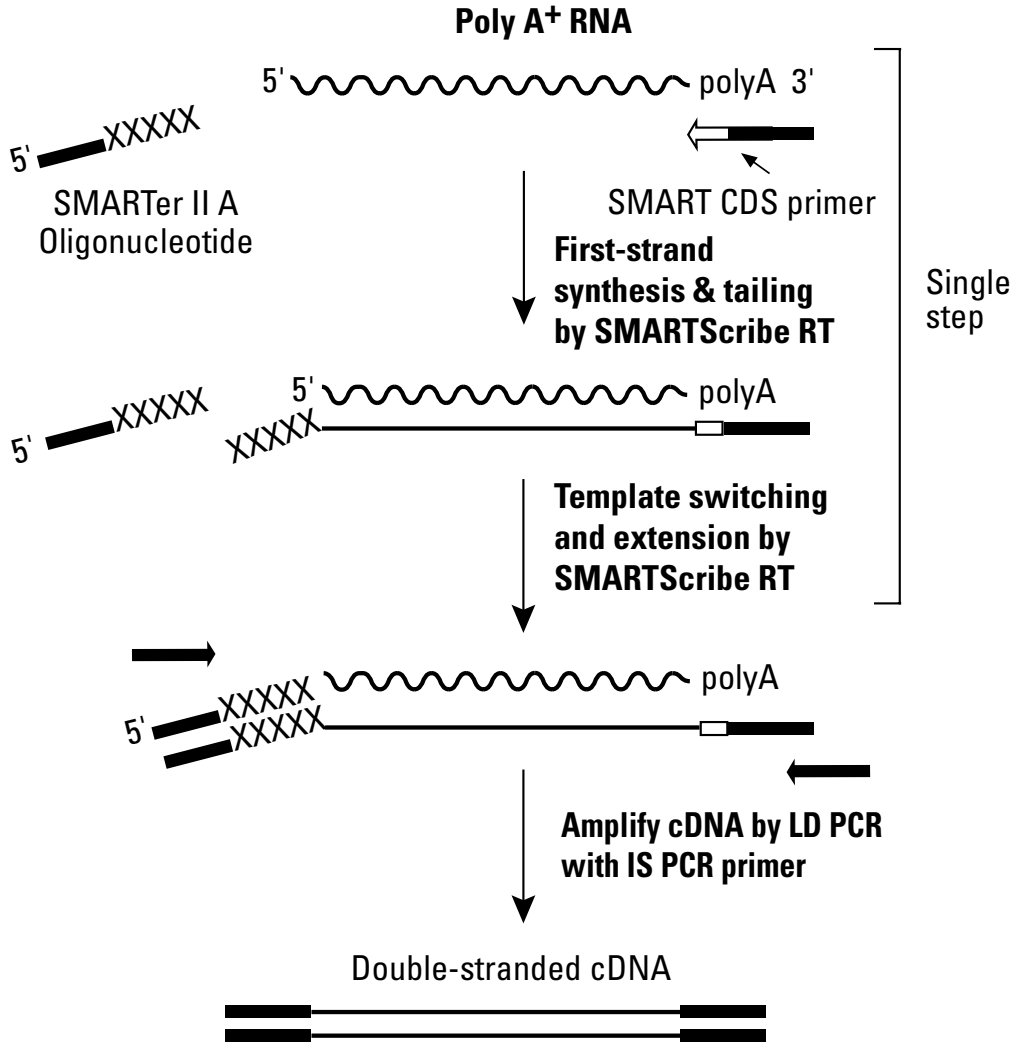


Figure 2. Flowchart of SMARTer cDNA Synthesis. The SMARTer II A Oligonucleotide, 3′ SMART CDS Primer II A, and IS PCR Primer all contain a stretch of identical sequence (see Section I for sequence information).

IV. SMARTer cDNA Synthesis

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

This protocol is optimized for the generation of cDNA starting from ultra-low amounts of total RNA using Clontech's SMART™ technology. The protocol also works starting from cells. Due to the sensitivity of the protocol, the input material (total RNA or cells) needs to be collected and purified under clean room conditions to avoid contamination. The whole process of SMARTer cDNA Synthesis should be carried out in a PCR Clean Work Station under clean room conditions (see Appendix A: PCR Clean Work Station Guidelines).

A. Requirements for Preventing Contamination

- **Before you set up the experiment, make sure you have two physically separated work stations. The first should be the PCR Clean Work Station (see Appendix A) for all Pre-PCR experiments that require clean room conditions such as first-strand cDNA synthesis (Section IV.E.) and purification of first-strand cDNA (Section IV.F). The second work station can be in the general laboratory where you will perform PCR, measure cDNA concentration, and work on any other experiments involving the PCR-amplified cDNA.**
- **It is absolutely required to have a PCR work station in a clean room with positive air flow, as contamination may occur very easily. Once contamination occurs it can be difficult to remove.**
- You need to strictly follow the clean room operation rules, i.e., you can only move materials/supplies from the clean room to the general lab, NOT the other way around. Don't share any equipment/reagents between the clean room and the general lab. You need a separate PCR machine inside the PCR workstation for cDNA synthesis.
- Wear gloves and sleeve covers throughout the procedure to protect your RNA samples from degradation by contaminants and nucleases. Be sure to change gloves and sleeve covers between each section of the protocol.

B. General Requirements

- **The success of your experiment depends on the quality of your starting sample of RNA. Prior to cDNA synthesis, please make sure that your RNA is intact and free of contaminants.**
- The assay is very sensitive to variations in pipette volume, etc. Please make sure all your pipettes are calibrated for reliable delivery, and make sure nothing is attached to the outside of the tips.
- All lab supplies related to SMARTer cDNA synthesis need to be stored in a DNA-free, closed cabinet. Reagents for SMARTer cDNA synthesis need to be stored in a freezer/refrigerator that has not previously been used to store PCR amplicons.
- Add enzymes to reaction mixtures last, and thoroughly incorporate them by gently pipetting the reaction mixture up and down.
- Do not increase (or decrease) the amount of enzyme added or the concentration of DNA in the reactions. The amounts and concentrations have been carefully optimized for the SMARTer amplification reagents and protocol.
- If you are using this protocol for the first time we strongly recommend that you perform negative & positive control reactions to verify that kit components are working properly.

IV. SMARTer cDNA Synthesis, *continued*

C. Sample Recommendations

The sequence complexity and the average length of SMARTer cDNA is noticeably dependent on the quality of starting RNA material. Due to the limiting sample size, most traditional RNA isolation methods may not be applicable.There are several commercially available products that enable purification of total RNA preparations from extremely small samples [e.g. Clontech offers the NucleoSpin RNA XS Kit (Cat. No. 740902.10) for purification of RNA from 10^2 cells]. When choosing a purification method (kit) ensure that it is appropriate for your sample amount.

Though SMART Technology is sensitive enough to generate cDNA from as little as 10 pg of total RNA, the use of a higher amount of starting material (100 pg to 10 ng) is recommended for reproducible amplification of low abundance mRNA transcripts.

After RNA extraction, if your sample size is not limiting, we recommend evaluating total RNA quality using the Agilent RNA 6000 Pico Kit (Cat. No. 5067-1513).

D. Sample Requirements

Total RNA

This protocol has been optimized for cDNA synthesis starting from 100 pg of total RNA. However, if your RNA sample is not limiting, we recommend that you start with more total RNA (up to 10 ng). Purified total RNA should be in nuclease-free water. If the total RNA volume is larger than 1 µl, perform ethanol precipitation and resuspend the pellet in 1 µl of nuclease-free water.

Cells

Although this protocol was optimized for cDNA synthesis starting from total RNA, the protocol has also been validated to work starting from cells. See **Appendix B:Working with Cells** for instructions on selecting the appropriate media and preparing your cells for first-strand cDNA synthesis.

E. PROTOCOL: First-Strand cDNA Synthesis *(perform in PCR Clean Work Station)*

IMPORTANT:
To avoid introducing contaminants to your RNA sample, the first part of the cDNA synthesis protocol (Sections E-G) requires use of a PCR work station in a clean room. Standard clean room procedure should be followed. If no clean room is available, you may work with just a PCR Clean Work Station on a temporary basis. We strongly recommend putting the PCR work station in a clean room to avoid contamination. It is critical to have an air blower in the PCR work station turned "on" during the whole process. Refer to Appendix A for PCR Clean Work Station Guidelines.

1. Prepare a stock solution of Reaction Buffer by mixing the Dilution Buffer with the RNase Inhibitor as indicated below, scale-up as needed:

19 µl	**Dilution Buffer**
1 µl	**RNase Inhibitor**
20 µl	**Total Volume**

IV. SMARTer cDNA Synthesis, *continued*

2. See Table I for guidelines on setting up your control & test samples. Transfer each whole volume of 3.5 µl to individual 0.2 mL RNase-free PCR tubes in an 8-well strip.

Table I: Sample Preparation Guidelines

Components	Negative Control	Positive Control	Test Sample
Reaction Buffer	2.5 µl	2.5 µl	2.5 µl
Nuclease-free water	1 µl	-	-
Diluted Control RNA*	-	1 µl	-
Sample	-	-	1 µl
Total Volume	3.5 µl	3.5 µl	3.5 µl

*The Control RNA is supplied at a concentration of 1 µg/µl. The Control RNA should be diluted in nuclease-free water to match the concentration of your test sample. Perform serial dilutions on the Control RNA until you obtain the appropriate concentration.

3. Place the samples on a –20°C prechilled IsoFreeze PCR rack in a PCR clean station, and add 1 µl of 3′ SMART CDS Primer II A (12 µM). Mix the contents and spin the tube(s) briefly in a microcentrifuge:

3.5 µl	Cell/Total RNA in Reaction Buffer (from Table I)
1 µl	**3′ SMART CDS Primer II A** (12 µM)
4.5 µl	**Total Volume**

4. Incubate the tube(s) at 72°C in a hot-lid thermal cycler for 3 min, then put the samples on the IsoFreeze PCR rack.

NOTE: The initial reaction steps (Step 5-7) are critical for first-strand synthesis and should not be delayed after Step 4. You can prepare your master mix (for Step 5) while your tubes are incubating (Step 4) in order to jump start the cDNA synthesis.

5. Meanwhile, prepare a Master Mix for all reactions plus one by combining the following reagents in the order shown at room temperature:

2 µl	**5X First-Strand Buffer**
0.25 µl	**DTT** (100 mM)
1 µl	**dNTP Mix** (10 mM)
1 µl	**SMARTer II A Oligonucleotide** (12 µM)
0.25 µl	**RNase Inhibitor**
1 µl	**SMARTScribe™ Reverse Transcriptase** (100 U)*
5.5 µl	**Total Volume added per reaction**

* Add the reverse transcriptase to the master mix just prior to use. Mix well by gently vortexing and spin the tube(s) briefly in a microcentrifuge.

6. Add 5.5 µl of the Master Mix to each reaction tube from Step 4. Mix the contents of the tube(s) by gently pipetting, and spin the tube(s) briefly to collect the contents at the bottom.
7. Incubate the tubes at 42°C for 90 min.
8. Terminate the reaction by heating the tube(s) at 70°C for 10 min.

IV. SMARTer cDNA Synthesis, *continued*

F. PROTOCOL: Purification of First-Strand cDNA using SPRI Ampure Beads *(perform in PCR Clean Work Station)*

The first-strand cDNA selectively binds to SPRI beads leaving contaminants in solution which is removed by a magnetic separation. The beads are then directly used for PCR amplification.

NOTE:

- Before use, beads should be brought to room temperature and mixed well to disperse.
- We recommend taping a 10 µl pipette tip refill rack onto the top of the MagnaBot Magnetic Separation device to keep the tubes/strips in place.

To purify the SMART cDNA from unincorporated nucleotides and small (<0.1 kb) cDNA fragments, follow this procedure for each reaction tube:

1. Add 25 µl of SPRI Ampure XP beads to each sample using a 200 µl pipetter. Adjust the pipetter to 35 µl, and pipette the entire volume up and down 10 times to mix thoroughly. The beads are viscous; suck the entire volume up, and push it out slowly. Incubate at room temperature for 8 minutes to let DNA bind to the beads.
2. Briefly spin the sample tubes to collect the liquid from the side of the wall. Place the sample tubes on the Promega MagnaBot II Magnetic Separation Device for 5 min or longer, until the solution is completely clear.
3. While samples are still on the Magnetic Separation Device pipette out the solution and discard. Briefly spin the tubes to collect the liquid at the bottom.
4. Place the tubes back in the Promega MagnaBot II Magnetic Separation Device for 2 min or longer to let beads separate from the liquid completely. Pipette out the residual liquid from the beads using a 10 µl pipetter and discard. Make sure that there is no supernatant remaining in the tube. **Be careful not to take out any beads with the supernatant.**

G. PROTOCOL: ds cDNA Amplification by LD PCR *(perform steps 1 & 2 in PCR Clean Work Station)*

We strongly recommend use of the **Advantage® 2 PCR Kit** (Cat. Nos. 639206 & 639207) for PCR amplification. The Advantage 2 Polymerase Mix has been specially formulated for efficient and accurate amplification of cDNA templates by long-distance PCR (LD PCR; Barnes, 1994). Table II provides guidelines for optimizing your PCR, depending on the amount of total RNA used in the first-strand synthesis.

IMPORTANT:
Optimal parameters may vary with different templates and thermal cyclers. To determine the optimal number of cycles for your sample and conditions, we strongly recommend that you perform a range of cycles. Do not exceed the recommended cycle numbers in the table below for different starting amounts of material. Refer to Appendix C for PCR optimization guidelines.

Table II: Cycling Guidelines Based on Amount of Starting Material

Input Amount, Total RNA	Input Amount, Cells	Typical No. of PCR Cycles
10 ng	1000 cells	12
1 ng	100 cells	12
500 pg	50 cells	13
100 pg	10 cells	15

IV. SMARTer cDNA Synthesis, *continued*

1. Prepare a PCR Master Mix for all reactions, plus one additional reaction. Combine the following reagents in the order shown, then mix well by vortexing and spin the tube briefly in a microcentrifuge:

5 µl	**10X Advantage 2 PCR Buffer**
2 µl	**dNTP Mix** (10 mM)
2 µl	**IS PCR Primer** (12 µM)
2 µl	**50X Advantage 2 Polymerase Mix**
39 µl	**Nuclease-Free Water**
50 µl	**Total Volume per reaction**

2. Add 50 µl of PCR Master Mix to each tube containing DNA bound to the beads from Section IV.F, Step 4. Mix well and briefly spin down.

IMPORTANT:
Transfer the samples from the PCR Clean Work Station to the general lab. All downstream processes will be performed in the general lab.

3. Place the tube in a preheated thermal cycler with a heated lid. Commence thermal cycling using the following program:

- 95°C 1 min
- X cycles[a]:

  95°C 15 sec
  65°C 30 sec
  68°C 6 min
- 72°C 10 min
- 4°C forever

[a]Consult Table II for guidelines. Determine the optimal number of PCR cycles using the protocol in **Appendix C: PCR Optimization**.

V. Amplified cDNA Purification & Validation

A. PROTOCOL: Purification of ds cDNA using SPRI Ampure Beads

PCR-amplified cDNA is purified by immobilizing it onto SPRI beads. The beads are then washed with 80% Ethanol and eluted in Purification Buffer.

1. Take out a 96-well Axygen V-bottom plate and cover all the wells with a MicroAmp Clean Adhesive Seal. Uncover only the wells that you want to use. Vortex SPRI beads till even, then add 90 µl of SPRI Ampure XP Beads to the wells of the 96-well plate.
2. Transfer the entire PCR product including the SPRI beads (from Section IV.G, Step 3) to the wells of the plate containing the SPRI beads (from Step 1 above). Pipette the entire volume up and down 10 times to mix thoroughly. Incubate at room temperature for 8 min to let the DNA bind to the beads.

   NOTE: The beads are viscous; suck the entire volume up, and push it out slowly.
3. Place the 96-well plate on the Ambion Magnetic Stand-96 for ~5 min or longer, until the liquid appears completely clear, and there are no beads left in the supernatant.
4. While the plate is sitting on the magnetic stand, pipette out the supernatant.
5. Keep the plate on the magnetic stand. Add 200 µl of freshly made 80% Ethanol to each sample without disturbing the beads to wash away contaminants. Wait for 30 seconds and carefully pipette out the supernatant. DNA will remain bound to the beads during the washing process.
6. Repeat Step 5 one more time.
7. Seal the sample wells on the plate and briefly spin down for 10 seconds at 1,000 rpm to collect the liquid at the bottom of the well.
8. Place the 96-well plate on the magnetic stand for 30 seconds, then remove all the remaining Ethanol.
9. Place the plate at room temperature for ~3-5 min until the pellet appears dry. You may see a tiny crack in the pellet.

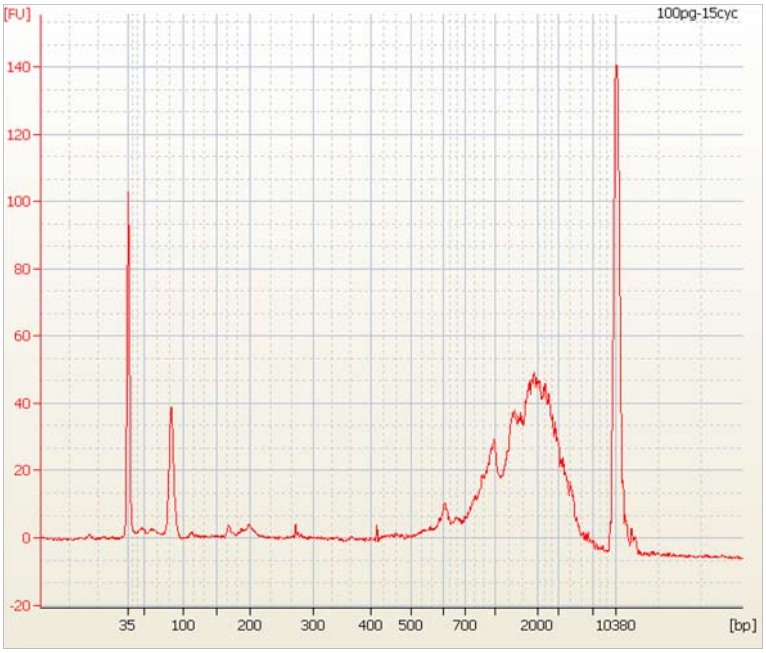
   NOTE: If you over-dried the beads, you will see many cracks in the pellet. If it is under-dried, the DNA recovery rate will be lower because of the remaining Ethanol.
10. Once the beads are dried, add 12 µl of Purification Buffer to cover the beads. Remove the plate from the magnetic stand and incubate at room temperature for 2 min to rehydrate.
11. Mix the pellet by pipetting up and down 10 times to elute DNA from the beads, then put the plate back on the magnetic stand for 1 minute or longer until the solution is completely clear.
12. Transfer clear supernatant containing purified cDNA from each well to a nuclease-free nonsticky tube. Label each tube with sample information and store at –20°C.

B. Validation Using the Agilent 2100 BioAnalyzer

1. Aliquot 1 µl of the amplified cDNA for validation using the Agilent 2100 BioAnalyzer and the High Sensitivity DNA Chip from Agilent's High Sensitivity DNA Kit (Cat. No. 5067-4626). See the user manual for the Agilent High Sensitivity DNA Kit for instructions: **http://www.chem.agilent.com/Library/usermanuals/Public/G2938-90320_HighSensitivityDNAQuick.pdf**
2. Compare the results for your samples & controls (see Figure 3) to verify whether the sample is suitable for further processing. Successful cDNA synthesis and amplification should yield no product in the negative control (Figure 3, Panel B), and a distinct peak spanning 400 bp to 9000 bp, peaked at ~2000 bp for the control RNA sample (Figure 3, Panel A), yielding approximately 2–7 ng of cDNA (depending on the input). Contaminated samples will have a broader peak, and abnormally high yield (Figure 3, Panel C).
3. Proceed to **Section VI: Covaris Shearing.**

V. Amplified cDNA Purification & Validation, *continued*

A



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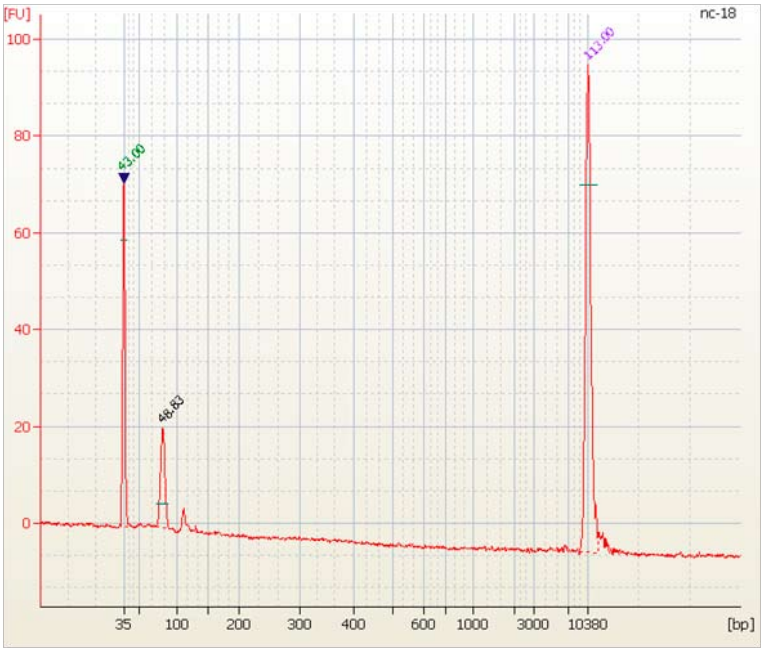

Figure 3. Electropherogram example results from Agilent 2100 Bioanalyzer. All samples were subjected to SMARTer cDNA synthesis and amplification as described in the protocol. FU = fluorescence absorption units. **Panel A.** Clean SMARTer Amplification Product (15 PCR cycles) **Panel B.** Clean SMART Negative Control (18 PCR cycles) **Panel C (see pg. 14).**

V. Amplified cDNA Purification & Validation, *continued*

C

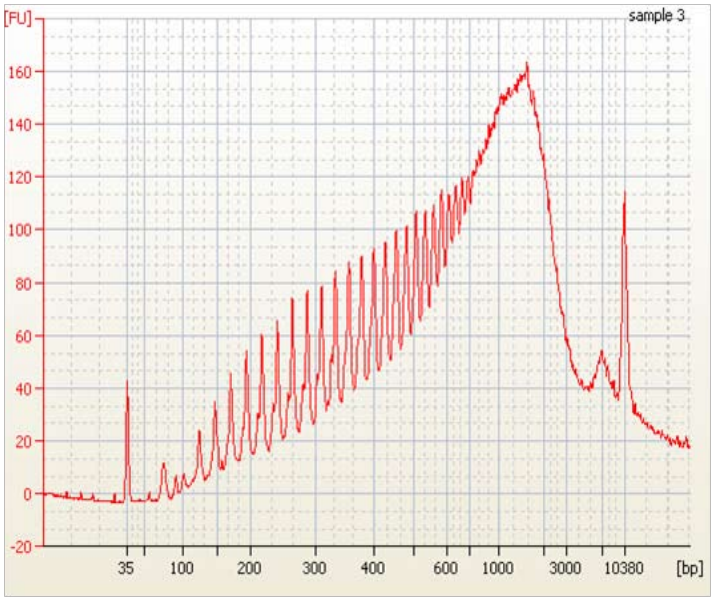

Figure 3 continued. Electropherogram example results from Agilent 2100 Bioanalyzer. Sample was subjected to SMARTer cDNA synthesis and amplification as described in the protocol. Panel C. Example of Contaminated SMARTer Amplification Product.

VI. Covaris Shearing

A. PROTOCOL: Covaris Shearing of Full-length cDNA

Prior to generating the final library for Illumina sequencing, the Covaris AFA system is used for controlled DNA shearing. The resulting DNA will be in the 200–500 bp size range.

1. Turn power ON for the Covaris system and the main cooler. Add about 1.9 L of distilled or deionized water to the water bath. The water level in the cooler should be within +/- 3 mm of the "FULL" waterline when the transducer is submerged. If needed, add distilled or deionized water to the water bath until the "FULL" line is reached.

IMPORTANT:
Never run a process without the water bath. This will permanently damage the transducer.

2. Close the door and open the Sonolab software. Click "ON" for the degassed button, and degas the water bath for 1/2 hour (30 minutes).
3. Add 65 µl of Purification Buffer to the DNA from Section V.A., Step 12. Transfer 75 µl of the Purification Buffer + DNA mixture into the 100 µl Covaris tube. Put the sample tubes into the appropriate location on the Sample holder.
4. Set up the process configuration panel based on the following table:

Table III. Process Configuration Panel Set Up

Duty %	Intensity	Burst cycle	Time (min)	Mode
10	5	200	5 min	Frequency Sweeping

5. Save the file and click return to go back to the main page.
6. Open the door. Place the tube holder with sample tubes on the transducer positioning system.
7. Close the door.
8. Click "START" on the main page to run the process.
9. After shearing is complete, transfer 75 µl of sheared DNA to 1.5 ml tubes.
10. Proceed to generate an Illumina Sequencing Library with the Illumina Paired-End DNA Sample Prep Kit (Illumina Cat. Nos. PE-102-1001 & PE-102-1002). Dispose all tubes and pipettes that have been exposed to amplicons in a sealed trash bag.

VII. Appendix A: PCR Clean Work Station Guidelines

A. Equipment Needed in the PCR Clean Work Station

IMPORTANT:
The following equipment must be kept in the PCR Clean Work Station only. Don't share any equipment/reagents between the clean room and the general lab. You can only move materials/supplies from the clean room to the general lab, NOT the other way around. Do not use equipment from the general lab for the portions of the protocol that require a PCR Clean Work Station.

- **Single channel pipette:** 10 µl, 20 µl and 200 µl, one each
- **Eight channel pipette:** 20 µl and 200 µl, one each
- **Filter pipette tips:** 10 µl, 20 µl and 200 µl, one box each
- **One dedicated PCR thermal cycler used only for first-strand synthesis**
- **One QuickSpin minicentrifuge for 1.5-ml tube & one QuickSpin minicentrifuge for 0.2-ml tube**
- **MagnaBot II Magnetic Separation Device** (Promega Part No. V8351)
- **One marker pen**
- **Small 1.5-ml tube rack**
- **Small bottle of nuclease-free water**
- **One bag of 1.5-ml nuclease-free tubes**
- **One bag of 8-strip nuclease-free 0.2-ml thin wall PCR tubes with caps**
- **DNA-OFF Solution** (Takara Cat. No. 9036)

B. PCR Clean Work Station Operation Instructions

IMPORTANT:
At the beginning of each section of the protocol listed below, be sure to put on a clean pair of gloves and sleeve covers, then turn on the light and blower on the PCR clean station and lift the cover window. Bring a clean trash bag into the PCR clean station as well as two pieces of kimwipes to cover the work area. When done using the PCR Clean Work Station (after Section IV.G.), wipe the working area dry with kimwipes, and clean with 70% EtOH. Once a week, clean the working area with DNA-OFF Solution.

Clean Work Station Instructions for First-Strand cDNA Synthesis (Section IV.E):

1. Bring the IsoFreeze PCR Rack and the Flipper Rack containing the SMARTer cDNA synthesis reagents from the –20°C freezer to the PCR clean work station, and put them on top of the kimwipes.
2. Take the cell or RNA samples from –80°C and put them on the IsoFreeze PCR Rack in the PCR clean work station.
3. Set up the cDNA synthesis reaction(s), and put the sample(s) in the PCR thermal cycler for cDNA synthesis.
4. Place all the used tips and tubes in a trash bag, remove from the PCR clean work station, and dispose.
5. Put the IsoFreeze PCR Rack and the Flipper Rack containing the SMARTer cDNA synthesis reagents back in the –20°C freezer.
6. Close the cover window of the PCR clean work station, leave the blower on, and dispose of gloves and sleeve covers.

VII. Appendix A: PCR Clean Work Station Guidelines, *continued*

Clean Work Station Instructions for Purification of fs cDNA & LD-PCR (Sections IV.F & IV.G):

7. Bring the Ampure XP beads from 4°C to the PCR clean work station. Aliquot the beads into 2 tubes: one for the first-strand cDNA purification (Section IV.F) and one for the PCR product purification (Section V.A) prior to the experiment to avoid a cross-contamination.
8. Bring the IsoFreeze Flipper Rack containing the SMARTer PCR reagents from the –20°C freezer to the PCR clean work station, and put it on top of the kimwipes.
9. Set up the PCR reactions.
10. Take the PCR reaction tubes out of the PCR clean work station, and perform PCR in a thermal cycler in the general lab.
11. Place used tips and tubes in a trash bag, remove from the PCR clean work station, and dispose.
12. Put the IsoFreeze Flipper Rack containing the SMARTer PCR reagents back in the –20°C freezer and put the Ampure XP beads back in the 4°C refrigerator.
13. Close the cover window of the PCR clean work station. Turn off the light and blower, and turn on the UV light.

VIII. Appendix B: Working with Cells

A. PROTOCOL: Screen for Cell Culture Media

IMPORTANT:
When working with cultured cells you need to ensure that the cell culture medium does not inhibit first-strand cDNA synthesis. Therefore it is important to select a medium with the least inhibitory effect. Please select cell culture medium according to the protocol below. Use the selected medium to perform serial dilution of your cells and picking of cells for cDNA synthesis.

1. Prepare 10 ng/µl & 100 pg/µl serial dilutions of the Control Total RNA and cell sample in Nuclease-Free Water, then dilute to 5 pg/µl with Reaction Buffer (for the positive control) or test medium (for the test sample). Collect as many media as possible in order to do one screening with a negative and positive control per test sample. See Table IV for guidelines.

Table IV: Media Screen Preparation Guidelines

Components	Negative Control	Positive Control	Test Sample
Reaction Buffer	2.5 µl	-	-
Nuclease-Free Water	1 µl	1 µl	-
Total RNA/Cell Suspension	-	2.5 µl (5 pg/µl)	2.5 µl (5 pg/µl)
Test Buffer/Medium	-	-	1 µl
Total Volume	3.5 µl	3.5 µl	3.5 µl

2. Perform the SMARTer cDNA synthesis protocol (Section IV.E-G) with samples and controls from Step 1 using 18 cycles of PCR.
3. Purify the PCR products with SPRI beads (Section V.A).
4. Run 1 µl of each sample from Step 3 on an Agilent High Sensitivity DNA Chip using the Agilent 2100 Bioanalyzer. See the user manual for the Agilent High Sensitivity DNA Kit for instructions: **http://www.chem.agilent.com/Library/usermanuals/Public/G2938-90320_HighSensitivityDNAQuick.pdf**
5. Compare the yield between samples diluted in culture media and nuclease-free water. Select the media that exhibits the least inhibitory effects for cell picking.

Table V: Validated Media*

Superblock (Pierce Cat. No. 37515)
0.1 mL of [DMEM F12 + Glutamax (Invitrogen Cat. No. 10565)] + 3.6 µl of 25% BSA (Invitrogen Cat. No. A10008-01)
For 1 L of PBS Buffer (0.2 micron filtered): 0.2 g of KCl 0.24 g of KH_2PO_4 (anhydrous) 8.00 g of NaCl 1.44 g of Na_2HPO_4(anhydrous) add dH_2O to 1 L

*We recommend testing the viability of your cells in the media listed in Table V; these media have been tested with this protocol. If your cells do not thrive in any of the media listed in Table V, screen other media per the instructions listed above.

VIII. Appendix B: Working with Cells, *continued*

B. Cell Preparation for First-Strand cDNA Synthesis

IMPORTANT:
The cDNA synthesis protocol has been tested with suspension cells without internal labeling. Any cell that has gone through fixation won't work (the RNA won't release efficiently).

1. Pick cell(s) in 1 µl of validated media (see Section A. Screen for Cell Culture Media), then transfer to a 0.2 mL RNase-free PCR tube containing 2.5 µl of Reaction Buffer. Put the tube(s) on dry ice immediately.

NOTE:

- The total volume of suspended cells should not exceed 1-2 µl. If necessary, you can spin down your cells and resuspend them in Reaction Buffer.
- If you choose to transfer your cell(s) in 2 µl of media, you need to use double the amount of Reaction Buffer (5 µl). You must also double the volumes for all of the components in the first-strand synthesis reaction (Section IV.E, Steps 1, 2 & 4), as well as double the volume of beads for the first SPRI purification (Section IV.F, Step 1).

2. Proceed with **Section IV.E: PROTOCOL: First-Strand cDNA Synthesis**. If you will not be performing cDNA synthesis immediately, store the tube on dry ice or at –80°C till use.

IX. Appendix C: PCR Optimization

If you have a sufficient amount of starting material (>1 ng total RNA), we recommend optimizing the PCR cycling parameters for your experiment. If you have a very limited amount of material or your sample is unique, use a similar source of RNA or cells to perform PCR cycle optimization prior to using the actual sample. Choosing the optimal number of PCR cycles ensures that the ds cDNA will remain in the exponential phase of amplification. When the yield of PCR products stops increasing with more cycles, the reaction has reached its plateau. Overcycled cDNA can result in a less representative sample. Undercycling, on the other hand, results in a lower yield of cDNA. The optimal number of cycles for your experiment is one cycle fewer than is needed to reach the plateau. Be conservative: when in doubt, it is better to use fewer cycles than too many.

To perform PCR cycle optimization, prepare several tubes containing an amount of RNA equal to your sample amount. Subject each tube to a different range of cycles. For example if you have 1 ng of RNA, subject one tube to the recommended a number of cycles. Subject the other two tubes to 2-3 cycles fewer or more than the first tube, i.e. 15, 12 (recommended for 1 ng), and 10 cycles.

1. Use the following program for thermal cycling:
   - 95°C 1 min
   - X cycles:
     95°C 15 sec
     65°C 30 sec
     68°C 6 min
   - 72°C 10 min
   - 4°C forever
2. Perform **Purification of ds cDNA using SPRI Ampure Beads** (Section V.A).
3. Run samples on an Agilent High Sensitivity DNA Chip using the Agilent 2100 Bioanalyzer to evaluate DNA output. See the user manual for the Agilent High Sensitivity DNA Kit for instructions: **http://www.chem.agilent.com/Library/usermanuals/Public/G2938-90320_HighSensitivityDNAQuick.pdf**.
4. Determine the optimal number of cycles required for each experimental and control sample. We recommend using the lowest PCR cycle number that generates enough material for Illumina library construction.
5. Apply the optimal number of PCR cycles to your sample material.

X. Appendix D: Troubleshooting Guide

Table VI: Troubleshooting Guide for SMARTer cDNA Synthesis & Amplification

PROBLEM	CAUSE	SOLUTION
No cDNA yield with the control RNA	Control RNA is degraded	The control RNA is vigorously tested for RNase contaminations and provided in high concentrations (1 µg/µl) to reserve its integrity. It is essential that any item that could contact the control RNA is RNase-free.
	Control RNA is too diluted	Make sure to use recently calibrated pipettes to accurately perform serial dilutions of the control RNA. Prepare fresh dilutions of the Control RNA and try again. The diluted RNA is less stable therefore avoid using previously diluted low concentration RNA samples.
	cDNA synthesis reaction failed	Carefully check the protocol and make sure all components are added in a right order; Failure of any components of the reaction will result in a failed cDNA synthesis. Make sure to prepare all the necessary items prior cDNA synthesis and to spin down tubes prior opening them. Do not delay cDNA synthesis reaction after RNA is denatured to jump start cDNA synthesis.
	PCR failed	Make sure to use the recommended PCR enzyme. Before using the PCR enzyme check it with previously tested primers and template.
	cDNA was not efficiently bound to the SPRI beads during purification steps	Make sure beads are mixed correctly after adding them to DNA samples. Vortexing the beads once they are added to samples can shear the DNA or break it free from the beads. Make sure the SPRI beads are collected correctly on the magnetic stand and given enough time to completely separate the liquid phase. Make sure not to disturb the beads while removing the supernatant. Supernatant that is not completely removed from SPRI beads may inhibit downstream reactions.
No cDNA yield with the experimental RNA, but control is OK	RNA is low quality, degraded or has impurities that inhibit the cDNA synthesis reaction	If possible, check RNA quality prior to cDNA synthesis. Make sure RNA is good quality. Certain tissues, such as plants, have a high level of polysaccharides that interfere with first-strand synthesis. Make sure to use appropriate RNA isolation kits for each given tissue/species.
Low cDNA yield with the control RNA	Control RNA is too diluted	Make sure to use recently calibrated pipettes to accurately perform serial dilutions of the control RNA. Prepare fresh dilutions of the Control RNA and try again. Diluted RNA is less stable therefore avoid using previously diluted low concentration RNA samples.
	cDNA was not efficiently bound to the SPRI beads during purification steps	Make sure the SPRI beads are collected correctly on the magnetic stand and given enough time to completely separate the liquid phase. Make sure not to disturb beads while removing the supernatant. Repeat cDNA synthesis with the freshly diluted control RNA.
	SPRI beads were overdried during purification of amplified cDNA	Don't allow the post-wash bead pellet to be overdried. It's important to allow any residual ethanol to dry, but overdrying (too long, or drying at a temperature higher than room temperature) will make it difficult to elute the DNA from the beads.

X. Appendix D: Troubleshooting Guide, *continued*

Table VI: Troubleshooting Guide for SMARTer cDNA Synthesis & Amplification...*continued*

PROBLEM	CAUSE	SOLUTION
Low cDNA yield with the experimental RNA, but control is OK	Insufficient amount of starting material or RNA is degraded or has impurities that inhibit the cDNA synthesis reaction	If more RNA sample is available, quantitate RNA prior to cDNA synthesis. Follow PCR cycle guidelines appropriate for the starting amount of sample you are using.

XI. References

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